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SIMULTANEOUS DETERMINATION OF SPERM CONCENTRATION AND VIABILITY BY FLOW CYTOMETRY

TECHNICAL FIELD

The invention relates to the simultaneous determination of sperm concentration and live sperm cells in a semen sample.

BACKGROUND OF THE INVENTION

Accurate and precise determination of sperm concentration is an important issue for the Artificial Insemination (AI) industry since it provides assurance to the studs and customers that the insemination doses contain the sperm numbers indicated. This is especially important in relation to exportation of semen from domesticated animal species (Foote, 1972; Fenton et al., 1990; Woelders 1990; Evenson et al., 1993; Donoghue et al., 1996).

It is known in the art to use counting chambers such as Makler™ (Sefi Medical, Haifa, Israel) or hemacytometers for routine sperm counts. It is, however, a disadvantage that multiple measurements are required to achieve an acceptable precision and accuracy. This procedure is time consuming and makes the counting chambers slow in use. Single hemacytometer counts are not highly accurate because of inherent errors in the technique, such as obtaining a subsample representative of the semen sample, etc, and furthermore, the process is highly dependant upon operator skills.

A technique widely used by the artificial insemination industry to determine sperm cell concentration is based on spectrophotometric measurement of turbidity (Woelders 1990; Evenson et al., 1993). However, this method requires a calibration of the spectrophotometer which is repeated at regular intervals since the performance of the instrument will vary over time, whereby this method is only as accurate as the calibration method. It is clearly a disadvantage of the spectrophotometer that the calibration is most often based on one of the counting chambers mentioned above, whereby the spectrophotometer method is only as accurate as the counting chamber methods mentioned above.

A further disadvantage of the spectrophotometer method is that debris, such as gel-particles in boar semen, increases the semen turbid whereby inaccuracies are introduced in the method since the distinguishing of debris from semen is very difficult (Woelders 1990) and the method is only used extensively because of the rapidity of the method and the fact that no other method works in a satisfactory manner for these species.

Semen from species without large gel-particles in the semen may be analyzed on electronic counters wherein particles having a specific particle size are counted (Parks et al., 1985). It is a disadvantage of the electronic counters that it is not possible to distinguish semen cells from debris having a particle size corresponding to the particle size of the semen cells. Since semen from most species contains a high number of particles having a size corresponding to the size of spermatozoa (i.e. cytoplasmic droplets) the resulting counts for individual ejaculates can be highly inaccurate (Parks et al., 1985).

The evaluation of semen quality in artificial insemination stations as well as in laboratories for human semen and the evaluation of semen quality for semen from laboratory animals is normally based on an evaluation of sperm motility using a phase contrast microscope with a heated stage (Woelders, 1990). Although this procedure is valuable to ensure that semen of very poor quality is excluded from artificial insemination, the method does not have a high precision or accuracy. Furthermore, the method is highly dependent on operator skills and large variations are observed in the results obtained by different operators.

It is also known in the art to use flow cytometers for a characterisation of the sperm (Szollosi et al., 1986; Takacs et al., 1987; Evenson et al., 1993). In the methods disclosed, the sperm has been killed and stained with propidium iodide, PI, to measure sperm concentration. Thereby, only sperm concentration is found. It is a disadvantage of the method that no indication of the amount of live sperm cells is provided.

In another method, only the dead sperm cells are stained by propidium iodide, whereafter light scattered from the unstained particles (including live sperm) is detected. This method has been used by Takizawa et al. (1994, 1995 and 1998) as

well as by Yamamoto et al. (1996). It is a disadvantage of this method that there is no discrimination between live spermatozoa and debris in the semen. Thereby, gel-particles, cytoplasmic droplets, debris and bacteria are likely to be included in the sperm count according to this method, thereby inflating the value of both the measured concentration and detected viability.

It is further known in the art to stain the sperm cells using SYBR-14 and propidium iodide, PI, (Fertilight™, Molecular Probes, Oregon, USA) and by means of flow cytometric analyses to asses the viability of the sperm (Garner et al., 1994; Garner et al., 1995; Donoghue et al., 1995; Garner et al., 1996a; Garner et al., 1996b; Garner et al., 1997a; Garner et al., 1997b Garner et al., 1997c; Maxwell et al., 1997; Maxwell and Johnson ,1997; Penfold et al., 1997; Songsasen et al., 1997; Thomas et al., 1997; Vetter et al., 1998; Thomas et al., 1998; Chalah and Brillard, 1998).

However, it is a disadvantage of this method that the SYBR-14 is slightly toxic to spermatozoa whereby the amount of live spermatozoa in a sample will decrease over even a short time. Since a staining time of 10 to 15 minutes has been used routinely, results are likely to be slightly inaccurate due to the decrease in live spermatozoa over time (Christensen and Stenvang, unpublished data). It is a further disadvantage of the method that the incubation of samples for staining is made at 36-37°C, which is highly inconvenient in routine work in laboratories or artificial insemination stations assessing semen quality.

In US Patent no. 4.559.309 a process for characterization of sperm motility and viability is disclosed. A sperm sample is stained with Rhodamine 123 and ethidium bromide and the fluorescence emissions of the sperm are measured by means of a flow cytometer. By measuring fluorescence emissions at green and at red wavelengths a measure correlated with sperm motility (green counts) and dead or putative dead cells (red counts), respectively, is obtained. Staining of a second sample with acridine orange provides a measure of the percentage of each type of cell present in sample, such as mature sperm, immature sperm and somatic cells, and further a measure of normality/abnormality of the sperm cells is provided.

It is a disadvantage of the disclosed process that an absolute measure of the semen concentration is not provided. It is a further disadvantage of the process that two different staining procedures are used to obtain a measure of sperm motility and dead or dying cells and a measure of the types of cells in the sample.

It is further known in the art to control the sample injection to electronic counters or flow cytometers so as to obtain a measure of the concentration. It is a disadvantage of these methods that the control of the sample injections is affected by differences between instruments and that calibration is needed at regular intervals. One example of such an instrument is Partec Sperm Cell Counter™ (Partec GmbH, Münster, Germany) where performance is unacceptable for assessment of sperm concentration in bovine semen (Dumont et al., 1996).

Further, it is known to use a standardised bead solution for absolute counting of the sperm concentration, the performance of this method was, however, found unacceptable for assessment of sperm concentration (Dumont et al., 1996).

It is a disadvantage of all of the above-mentioned methods that only the ratio of live sperm cells or the concentration of the dead or killed sperm cells is determined. To obtain a determination of the ratio of live cells and a determination of the concentration of the semen an evaluation of the semen sample by at least two different methods is needed. It is a disadvantage of the above-mentioned methods that a single method determining the sperm concentration as well as the ratio of live sperm cells is not provided.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for a simultaneous determination of the concentration of sperm cells and the percentage of the live sperm cells in a semen sample.

It is a further object of the present invention to provide a method according to the above-mentioned method wherein the staining process is fast and made at room temperature.

The method of the invention for the determination of the concentration of sperm cells in a semen sample and the percentage of live sperm cells therein comprises subjecting the sample or an aliquot thereof to selective staining of live and dead sperm cells and combination with an internal concentration standard means and determining the concentration of the sperm cells and the percentage of live sperm cells simultaneously by means of a detection means responsive to the selective staining and the internal concentration standard means.

The information represented by the concentration of sperm cells and the percentage of live sperm cells constitutes a relevant set of data, but it will be understood that this set can, of course, be expressed as the concentration of live cells, or the concentration of dead cells, or other parameter calculable on the basis of the combination of the concentration of sperm cells and the percentage of live sperm cells.

Irrespective of the manner in which the data set is expressed, it may be used for the routine evaluation of semen for artificial insemination and for determination of the degree of dilution required for securing an adequate number of live cells in each insemination dose.

Usually, an aliquot of the sample is subject to the determination. As semen samples are relatively viscous, it is preferred that the aliquot of the semen sample is diluted to form a diluted subsample. The dilution of the sample is preferably performed using a diluent which sustains viability of the sperm cells during the determination.

The internal concentration standard means may be any concentration standard means that can be suitably combined with the sample or subsample and detected by the detection means to function as a reference indicative of the concentration of the sperm cells. The internal concentration means is suitably constituted by standardisation particles of a predetermined number which are combined with the subsample.

The selective staining of the live and dead sperm cells further permits for the determination of the percentage of dying cells. It is thus possible to discriminate

between live cells and dying cells. While conventional motility determinations performed by microscopy do not permit such a distinction, it is in the method of the invention possible to use a staining which will permit a determination of a transition phase during which the plasma membrane of the sperm cells become increasingly permeable as an indication of the cells being subject to deterioration. Such cells will appear motile in microscopic motility observations, but will have a much shorter active motile period than cells having an intact plasma membrane (Christensen et al., unpublished data).

The selective staining may be performed by using one or more fluorochromes resulting in fluorescent qualities being conferred to live cells and dead cells and, where applicable, to dying cells, the fluorescent quality or qualities of live cells being distinguishable, by the determination means, from the fluorescent quality or qualities of dead cells (or, where applicable, dying cells), the standardisation particles being fluorescent particles having a fluorescent quality distinguishable from the fluorescent qualities of the live, dead and/or dying cells, the determination being performed by selective counting of cells/particles of each fluorescent quality.

The detection means responsive to the fluorescent qualities of the live, the dead, and the dying cells as well as the fluorescent quality of the standardisation particles may comprise any fluorescent activated cell sorter, such as a flow cytometer, or a laser scanning cytometer, etc.

The fluorochromes staining the cells may be fluorochromes binding to DNA. Furthermore, the fluorochrome staining the dead or dying cells may be a fluorochrome capable of entering a cell through a leaking or defect plasma membrane, but substantially incapable of entering a cell having an intact plasma membrane, and the other fluorochrome is a fluorochrome capable of entering a cell through an intact cell membrane.

Dying cells have a leaking or non-intact plasma membrane whereby these cells are stained by the fluorochromes staining the live cells and also by the fluorochromes staining the dead cells. The staining of the dead cells will however be much stronger

than the staining of the dying cell, whereby the intensity of the staining provides information about the state of the cell.

The cells having a leaking plasma membrane will usually die shortly after this process has started. The number of dying cells is usually highest when a semen sample has been frozen or has been stored for a period prior to evaluation. Therefore, this method may also be used for development and evaluation of new media for freezing the sperm and new storage and/or freezing/thawing procedures.

The excitation of the fluorochromes may be performed by means of light in the wavelength range about 488 nm, the fluorochrome staining the live cells may be SYBR-14, and the fluorochrome staining the dead or dying cells may be propidium iodide, PI.

Alternatively, the excitation of the fluorochromes may be performed by means of light in the wavelength range about 543 nm, the fluorochrome staining the live cells may be MPR71292, and the fluorochrome staining the dead or dying cells may be ethidium-homodimer-2, EHD2.

The staining of the cells may be performed at a temperature below 35°C, such as at a temperature between 15°C and 25°C, preferably at room temperature, such as at 20°C.

The fluorochrome staining of the live cells may be used in concentrations below standard concentrations conventionally applied for such fluorochromes, such as in concentrations in the range from 25 to 75 nanomolar, preferably such as in concentrations about 50 nanomolar.

The standardisation particles used in combination with the semen sample may be fluorescent beads.

Furthermore, the size and concentration of the subsample may be adapted so that the number of sperm cells corresponds to between one tenth and ten times the number of standardisation particles, such as to between one quarter and four times the number

of standardisation particles, such as to between half and twice the number of standardisation particles.

The beads may be provided in a suspension comprising beads and diluent. It is an advantage of a suspension comprising both the beads and the diluent that the suspension may be manufactured by a manufacturer in a highly automated process to obtain a very accurate number of beads in the suspension. Furthermore, the suspension comprising the diluent and the beads may be manufactured in tubes, the tubes being suitable as measuring chambers in fluorescent activated cell sorters, such as flow cytometers. Thereby, inaccuracies originating from redistribution and dilution of the suspension are minimised. Still further, by using tubes from the same manufacturing process with the same lot number, corresponding results may be obtainable independently of different apparatuses being used by different users. For example, Sperm Count tubes CD4/3 and CD8/3 manufactured by Becton Dickinson containing a predetermined number of beads in a diluent may be used. Furthermore, tubes containing only beads, such as "TruCount" tubes manufactured by Becton Dickinson and containing a predetermined number of beads, may be used.

The diluent may be a diluent which is non-toxic to sperm and which sustains viability during the staining and analysis procedures. The diluent may be any such medium capable of preventing sperm cells from sticking to the side walls of the measuring chamber or measuring tube, such as a chemical compound, such as a protein, such as BSA, such as polyvinylalcohol (PVA), etc.

In order to obtain an even higher accuracy of the measurements, automated dilution of the semen may be applied. Thereby, the operator dependant part of the process is eliminated which makes the process highly reproducible.

Furthermore, the determination of the concentration of the sperm cells and the percentage of live sperm cells may be determined as a mean value of the determination of the concentration of the sperm cells and the percentage of live sperm cells performed at two or more subsamples of the semen sample. Hereby, the concentration of the sperm cells and the percentage of live sperm cells may be determined more accurate.

After the analysis of the subsample, the sample may be diluted to a predetermined semen concentration or a predetermined number of live sperm cells and distributed so as to fill one insemination dose to straws to be used with artificial insemination. An indication of the quality of the semen based on the semen concentration and the number of live sperm cells in the corresponding ejaculate may accompany the semen straws. Thereby, the users, such as the artificial insemination stations, may obtain valuable information regarding the semen.

Furthermore, it is possible to reject semen from ejaculates not fulfilling specified quality indications so as to ensure that only semen of a certain quality is used for artificial insemination. Still further, a price difference according to the quality indication of the semen may be applied.

The method of the invention may also be used to determine the absolute number of sperm cells produced by animals subjected to toxicology or other pharmacological experiments. Furthermore, the method of the invention may be used to prove that animals or human beings have been influenced by e.g. a toxicological environment or chemical invasions into the body.

Furthermore, because of the objectiveness and reproducibility of the method, the method may be used to evaluate the semen quality and quantity of human semen, both in connection with artificial insemination and *In vitro* fertilization and in connection with investigations of the proposed decline in human semen quality.

BRIEF DESCRIPTION OF THE DRAWING

Figs. 1 and 2 show print-outs for the assessment of sperm concentration on the Sysmex particle counter. A Difference between the first discrimination (arrows) and the second discrimination (dotted vertical line) led to differences in sperm concentration of 750 x 10^6 sperm/ml and 700 x 10^6 sperm/ml, respectively. Note that the Sysmex is designed for hematology counting and that the sperm is counted in the canal for red blood cells (RBC). The x-axis indicate the size of the particle according to

the Coulter principle and the curve illustrates the distribution of particles in relation to size,

Fig. 3 shows the regression line for FACSCount with blue laser (488 nm) against Makler Chamber. The data are corrected data from the measurement error model,

Fig. 4 shows the regression line for FACSCount with green laser (543 nm) against Makler Chamber. The data are corrected data from the measurement error model,

Fig. 5 shows the regression line for Spectrophotometer (L'Aiglon) against Makler Chamber. The data are corrected data from the measurement error model,

Fig. 6 shows the regression line for particle counter (Sysmex) against Makler Chamber. The data are corrected data from the measurement error model,

Fig. 7 shows the regression line for FACSCount with green laser (543 nm) against FACSCount with blue laser (488 nm). The uncorrected data show a very high correlation between these techniques. The slope β is 0.99 and the correlation coefficient is 0.99.

EXPERIMENTAL

Summary: Two FACSCount flow cytometers (BDIS Europe, Erembodegem-Aalst, Belgium) equipped with either blue (488nm) or green laser (543nm) have been tested with regard to simultaneous determination of sperm concentration and viability in a bull AI station. Samples from 52 ejaculates of 25 bulls were diluted 250 x in a PBS medium containing 0.1% BSA and a 20 µl aliquot was transferred to a Sperm Count tube containing approximately 100,000 beads. Sperm was stained with a combination of SYBR-14 and PI for analysis with blue excitation (488 nm) or a combination of MPR71292 and EHD2 for analysis with green excitation (543nm).Then, data from the FACSCounts were analysed with Attractor™ software (BDIS Europe, Erembodegem-Aalst, Belgium). The sperm concentration was also determined with a spectrophotometer (L'Aiglon, IMV, Cedex, France) and a particle counter (Sysmex F-820, SYSMEX, Almind, Denmark). The four methods were compared to manual

counting using a Makler chamber (Sefi Medical, Haifa, Israel) and a phase contrast microscope. In addition to the determination of sperm concentration, the flow cytometric analyses also provided an estimate of sperm viability.

In general, a good agreement was found between the four methods and the Makler chamber. The short staining period (2½-5 min) and the reduced toxicity of the dyes used (the dyes in the concentrations used should not be toxic within a 10 min staining time) makes it possible to determine sperm viability with high precision and accuracy.

MATERIALS AND METHODS

Semen samples:

The semen samples to be used in the experiments were collected from 25 bulls giving a total of 52 ejaculates. The semen samples were collected during 4 days from bulls with differences in both semen concentration and quality. An aliquot of 2-3 ml of the raw semen was placed in a tube (NUNC Intermed Cat# 347880, Lifetechnologies, Roskilde, Denmark) and placed on a Swelab-820 mixer (Bie & Berntsen, Rødovre, Denmark) and mixed continuously. All processing and analyses were carried out within 30 minutes after semen collection.

Determination of sperm concentration by Makler chamber:

Two samples of the raw semen were diluted 1:40 with a Hamilton Microlab A503 autodiluter (Struers KEBO lab., Albertslund, Denmark) and using a phosphate buffered saline (PBS, pH 7.4, 300 mOsm with 0.1 % BSA). The diluted samples were placed on a mixer (Adams Nutator, NC. Nielsen, Brøndby, Denmark). Prior to evaluation, spermatozoa were immobilized by adding 7.5 µl 4% (w/v) formaldehyde and the Makler chamber was loaded with a first 7.5 µl subsample. The lid of the Makler chamber was placed in position and touched gently with the tip of a fingernail to ensure that it rested firmly on the pillars (Newton rings). A total of 25 squares was counted under phase contrast microscopy using 200 x magnification. After counting, the chamber was washed in distilled water, dried and reloaded with a subsample from

the same sample. Subsequently, this procedure was applied for the second subsample and a total of 4 measurements (2 samples x 2 replicates) was obtained per ejaculate.

Determination of sperm concentration by spectrophotometer:

A 20 µl sample of the raw semen was diluted to a total volume of 4 ml (1:200) in a cuvette for the L'Aiglon spectrophotometer. Dilution was done with a CAVRO autodiluter (Z 069, IMV). Following dilution, a small piece of vitawrap was placed over the cuvette and the diluted sample was mixed gently by hand. After resting approximately 10 sec., the cuvette was placed in the spectrophotometer and the sperm concentration was measured. The cuvette was re-covered by vitawrap and mixed gently by hand, and after a resting period the cuvette was re-measured in the spectrophotometer. Following this measurement, a second sample was diluted and measured. Thus, a total of 4 measurements was obtained per ejaculate.

Determination of sperm concentration by particle counter:

A two-step dilution is necessary in order to count sperm concentration on the Sysmex F-820 particle counter. The first step was dilution of 20 µl raw semen to a volume of 10 ml (1:500) and in the second step, 100 µl of the diluted semen was diluted to a total volume of 10 ml and placed in a sample-cup (1:100, resulting in a combined dilution of 1:50,000). Both dilution steps were performed with an AD-270 autodiluter (SYSMEX, Almind, Denmark). The sample-cup was covered with a small piece of vitawrap and mixed gently by hand, rested approximately 10 sec. and counted on the Sysmex. After counting, the sample-cup was re-covered with vitawrap, re-mixed and counted again. Then the sample was then discarded and a second sample was diluted, treated and counted according to this procedure. A total of 4 counts was obtained per ejaculate.

Determination of sperm concentration and viability by flow cytometer:

A two-step dilution is necessary for the flow cytometric analyses. The first step is dilution of a 20 µl sample to a total volume of 5 ml in a tube using the PBS medium described above (NUNC Intermed Cat# 3478880, Lifetechnologies, Roskilde, Denmark). From this tube, a 20 µl sample was transferred to a Sperm Count tube using reverse manual pipetting. The dyes were added to the Sperm Count tubes less than 12 hours before using the tubes in the analysing procedure. Two different flow

cytometers were used, one being a FACSCount with a green laser (543 nm) and the second being a FACSCount with a blue laser (488 nm). 2 µl of MPR71292 and 2µl of EHD-2 were added to the tubes to be analysed in the first FACSCount (543 nm) to provide for a final concentration of MPR71292 at 100 nM and a final concentration of EHD-2 at 5µM. 5 µl of SYBR-14 and 5µl of PI were added to the tubes to be analysed in the second FACSCount (488 nm) to provide for a final concentration of SYBR-14 at 50 nM and a final concentration of PI at 12µM. Prior to opening the Sperm Count tube, the tubes were whirlmixed for 3 sec in an upside-down position and for 3 sec in a normal position. Whirlmixing were repeated for two sec after addition of the sperm and before each analysis. After addition of sperm, the Sperm Count tubes were placed on a mixer (Adams Nutator, NC. Nielsen, Brøndby, Denmark) for 4 min at room temperature with no light reaching the tube. After 4 min, the first replicates were analysed simultaneously on the two flow cytometers and the second replicates were analysed after a 6 min staining period. Tubes were analysed twice to provide for a second replicate rather than pipetting two subsamples from the tubes. A total of 4 analysis was carried out on each flow cytometer per ejaculate.

Analysis of FACSCount data:

During the flow cytometric analyses, data were stored on a floppy disk in the FACSCount, and after the measurements, data from the floppy disk were analysed with Attractor™ (BD) software at the Royal Veterinary and Agricultural University, Section for Reproduction.

Statistical analysis:

Data obtained from the five methods (Makler chamber, spectrophotometer (L'Aiglon), particle counter (Sysmex), flow cytometer (FACSCount with green or blue laser) were subject to analyses of variance to point out significant effects of date, bull, sample, replicate, combinations of these effects as well as residual variation. Viability data from the FACSCount analyses were also subject to analyses of variance. The following statistical model (1) was used:

$$(1) \qquad Y_i = \alpha_{i,date} + B_{i,bull} + C_{i,bull*date} + D_{i,date*bull*sample} + E_i$$

where: Y_i = result for the method

$\alpha_{i,date}$ = effect of date (systematic)

$B_{i,bull}$ = effect of bull

$C_{i,bull*date}$ = effect of ejaculate

$D_{i,date*bull*sample}$ = effect of sample

ε_i = residual error

i = the number of observation (measurement), 1,...,208

Capital letters in the above model indicate random effects. However, estimates for overall sample and replicate differences were made in a statistical model with systematic effect of sample, replicate and date.

Data from the four methods (spectrophotometer, particle counter, flow cytometer with green or blue laser) were subject to regression analyses to compare the results of these methods with results obtained with the Makler chamber. Regression analyses were based on average values for the four measurements per ejaculate for each of the methods. At first, regression analyses were made without corrections, but due to the large variation of the Makler chamber data, regression analyses were also made using a measurement error model which takes the imprecision of the Makler chamber into account. All statistical analyses were performed with SAS version 6.12 (SAS, 1990).

Makler chamber:

The data obtained with the Makler chamber appeared more imprecise than anticipated from earlier data and the coefficient of variation was 21.3 %. The reason for the increased variation is unclear.

Precision of the spectrophotometer method:

The results of the analyses of variance for data obtained with the spectrophotometer are shown in table 1. The instrumental coefficient of variation (CV%) is 2.10 % and the large variation between samples within ejaculates (sample variation) make the method significantly more imprecise, and the 95% confidence interval for determination of sperm concentration with the L'Aiglon spectrophotometer is ± 169 x 10^6 sperm/ml when a single measurement is made (one sample, one measurement). A

small overall variation in concentration was detected between the two samples with sample 1 being 48.5 ± 15 x 10^6 sperm/ml lower than sample 2. The reason for this difference is unclear.

Precision of the particle counter method:

Table 1 also shows the results for the Sysmex particle counter. The coefficient of variation with this method is high, 6.1%. In the measurements two sets of counts were used where the discrimination on particle size differed in the two replicates, respectively, in- and excluding a population of smaller particles in the "sperm count" (see Figs. 1 and 2). If these observations are excluded, the coefficient of variation for the Sysmex is 4.0 %. The sample variation with the Sysmex particle counter is small and the 95% confidence interval of the method for a single measurement per ejaculate is ± 118 x 10^6 sperm/ml (two sets of counts mentioned above are excluded in this calculation, if included the 95% confidence interval is ± 182 x 10^6 sperm/ml).

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Table 1. Sources of variation for the four methods: spectrophotometer (L'Aiglon), particle counter (Sysmex) and FACSCount (green laser) and FACSCount (blue laser). Numbers in brackets indicate the percentage of the total variation for the methods.

Source of variation	L'Aiglon		Sysmex		FACSCount (green laser)		FACSCount (blue laser)	
Bull[1]	179,312	(52)	197,497	(51)	220,714	(58)	226,901	(59)
Bull*date[2]	155,884	(45)	186,918	(48)	153,674	(40)	151.766	(39)
Bull*date*sample[3]	6.462	(1.9)	64	(~0)	4,896	(1.3)	1,554	(0.4)
Residual[4]	968	(0.3)	3,599	(0.9)	3,755	(1.0)	7.285	(1.4)
CV%[5]	2.10		4.0*		4.3		6.0	
95% confidence int.[6]	± 169 x 10^6		± 118 x 10^{6**}		± 182 x 10^6		184 x 10^6	

[1]Overall variation between bulls

[2]Variation between ejaculates within bulls. (ejaculate variation)

[3]Variation between samples (sample variation)

[4]Residual variation (instrumental variation)

[5]Instrumental coefficient of variation

[6]95% confidence interval for a single measurement per ejaculate (one sample, one replicate)

*6.1 % if all measurements are included

**± 182 x 10^6 sperm/ml if all measurements are included

Precision of the particle counter method:

Table 1 also shows the results for the Sysmex particle counter. The coefficient of variation with this method is high, 6.1%. In the measurements two sets of counts were used where the discrimination on particle size differed in the two replicates,

respectively, in- and excluding a population of smaller particles in the "sperm count" (see Figs. 1 and 2). If these observations are excluded, the coefficient of variation for the Sysmex is 4.0 %. The sample variation with the Sysmex particle counter is small and the 95% confidence interval of the method for a single measurement per ejaculate is ± 118 x 10^6 sperm/ml (two sets of counts mentioned above are excluded in this calculation, if included the 95% confidence interval is ± 182 x 10^6 sperm/ml).

Precision of the FACSCount with green laser (543 nm):

The results of the FACSCount with green laser is shown in table 1. The coefficient of variation is 4.3 % and the 95% confidence interval for a single measurement per ejaculate is ± 182 x 10^6 sperm/ml. It should be noted that no significant differences are observed between replicates or samples.

Precision of the FACSCount with blue laser (488 nm):

The results of the FACSCount with blue laser (table 1) have a higher coefficient of variation (6.0 %) but due to less variation between samples the 95% confidence interval for a single measurement per ejaculate is ± 184 x 10^6 sperm/ml. A higher sperm concentration was observed for replicate 2 (54.7 ± 11 x 10^6 sperm/ml higher than replicate 1). The reason for this difference was unclear.

Precision of the FACSCount (green laser) for determination of sperm viability:

The results for determination of sperm viability with FACSCount (green laser) are shown in table 2. Coefficient of variation for the method is 1.2 %. The 95% confidence interval for determination of sperm viability based on one measurement per ejaculate is ± 4.0 %. A small decrease in sperm viability over time was observed (- 0.27 ± 0.07 %/min). This decrease is much less than decreases observed in trials using a staining time of more than 10 minutes. With a staining time of 3-5 minutes, the accuracy of the method is only affected slightly.

Precision of the FACSCount (blue laser) for determination of sperm viability:

The results of the FACSCount (blue laser) for determination of sperm viability are shown in table 2. The coefficient of variation is equal (CV = 1.1%) to that of the FACSCount with green laser but due to a slightly larger sample variation, the 95 % confidence interval for a single measurement per ejaculate is 6.2 %. Sperm viability

did not decrease over time (- 0.15 ± 0.07 %/min which are a likely effect of the reduced concentration of the SYBR-14 stain (50 nM)).

Table 2. *Sources of variation for flow cytometric determination of sperm viability.* Numbers in brackets indicate the percentage of the total variation of the method.

	FACSCount (green laser)		FACSCount (blue laser)	
Bull[1]	29.73	(65.8)	24.16	(45.8)
Bull*date[2]	11.24	(24.9)	18.75	(35.5)
Bull*date*sample[3]	3.26	(7.2)	9.01	(17.0)
Residual [4]	0.94	(2.1)	0.83	(1.6)
CV%[5]	1.2		1.1	
95% confidence int.[6]	± 4.0 %		± 6.2 %	

[1]Overall variation between bulls
[2]Variation between ejaculates within bulls. (ejaculate variation)
[3]Variation between samples within ejaculates (sample variation)
[4]Residual variation (instrumental variation)
[5]Instrumental coefficient of variation
[6]95% confidence interval for a single measurement per ejaculate (one sample, one replicate)

REGRESSION ANALYSES

The regression lines for the four methods after correction for imprecision of the "golden standard" are shown in Figs. 3 to 6 and the results of the regression analyses are shown in table 3. The slopes of all regression lines are not significantly different from 1 and the intercepts for the different regression lines are not significantly different from 0. It appears from table 3, that the error of the FACSCount analyses are

higher than for the spectrophotometer and particle counter. Correlation coefficients from the regression analyses without correction showed a slightly lower correlation for the analyses with FACSCount where the correlation for respectively the green and blue laser were 0.91 and 0.9 versus 0.945 for particle counter and 0.96 for spectrophotometer. This is in contrast to results obtained earlier where the correlation coefficients for respectively particle counter, spectrophotometer and FACSCount were 0.93, 0.93 and 0.97. In theory, the correlation for the FACSCount (either laser) should be higher than those of the other methods because the flow cytometric method identifies sperm from a staining of DNA. However, since bull semen contains a very little amount of debris, the difference between flow cytometry and the other techniques appears small.

GENERAL DISCUSSION

Sperm viability: The two methods for determination of sperm viability are highly accurate and only for the combination of 292 and EHD2 a slight decrease in viability (-0.27 ± 0.07 %/min) was observed. Since analyses can be performed after a 2½ min staining time, viability can be determined precisely as well as accurately. It may be desirable to stain two samples per ejaculate since sample difference is a significant source of variation. With two samples per ejaculate, the 95% confidence interval for the determination of sperm viability will be from 2.8 % (green laser) to 4.4 % (blue laser).

Table 3. Results of the regression analyses (corrected for imprecision of the Makler chamber). Values ± standard deviation (sd).

Method	intercept (α)	slope (β)	error
FACSCount (green laser)	- 150 ± 115	1.03 ± 0.07	70
FACSCount (blue laser)	- 200 ± 130	1.05 ± 0.08	70
Spectrophotometer (L'Aiglon)	20 ± 75	0.94 ± 0.05	40
Particle Counter (Sysmex)	-50 ± 85	1.01 ± 0.05	40

Sperm concentration: Determination of sperm concentration with FACSCount (either laser) is in the present investigation very close to the results achieved by the use of the particle counter (Sysmex) or the spectrophotometer (L'Aiglon) and the correlation between FACSCount methods are very high (Fig. 7). In both flow cytometric methods 5000 events were sampled per analysis, and it is believed that still better results may be obtained by a sampling of 10000 events.

A significant source of variation for the flow cytometric determination of sperm concentration is the difference between samples. This difference relates both to true difference between samples and to difference between Sperm Count tubes. The variation between samples may be reduced by applying a fully automated dilution procedure thereby reducing the operator dependent factor to a minimum. Furthermore, the results of the method may be improved by a determination of the sperm concentration for an ejaculate based on a mean value obtained from analyses of two Sperm Count tubes. Thereby, also the influence of variation in bead number between individual Sperm Count tubes could be reduced, and furthermore the sperm viability would be determined more precisely if it was based on two samples.

REFERENCES

Donoghue AM, Garner DL, Donoghue DJ, Johnson LA, 1995: Viability Assessment of Turkey Sperm Using Fluorescent Staining and Flow Cytometry. Poultry Scl, 74:1191-1200.

Donoghue AM, Thistlethwaite D, Donoghue DJ, Kirby JD, 1996: A New Method for Rapid Determination of Sperm Concentration in Turkey Semen. Poultry Sci., 75:785-789.

Dumont P, Coupet H, Gary F, 1996: Some aspects of evaluation of semen quality and processing in France. Proc. 8th European AI-Vets meeting.

Evenson DP, Parks JE, Kaproth MT, Jost LK, 1993: Rapid Determination on Sperm Cell Concentration in Bovine Semen by Flow Cytometry. J Dairy Sci 76:86-94.

Fenton SE, Ax RL, Cowan CM, Coyle T, Gilbert GR, Lenz RW: Validation and Application of an Assay of Deocyribonucleic Acid to Estimate Concentrations of Bull Sperm. J. Dairy Sci., 73:3118-3125.

Foote RH, 1972: How to Measure Sperm Cell Concentration by Turbidity (Optical Density). Proceedings 4th Techn. Conf. on Anim. Reprod. and AI, NAAB, pp. 2-6.

Garner DL, Johnson LA, Yue ST, Roth BL, Haugland RP, 1994: Dual DNA Staining Assessment of Bovine Sperm Viability Using SYBR-14 and Propidium Iodide. J Androl,15:620-629.

Garner DL and Johnson LA, 1995: Viability Assessment of Mammalian Sperm Using SYBR-14 and Propidium Iodide. Biol Reprod, 53:276-284.

Garner DL, Dobrinsky JR, Welch GR, Johnson LA, 1996a: Porcine Sperm Viability, Oocyte Fertilization and Embryo Development after Staining Spermatozoa with SYBR-14. Theriogenology, 45:1103-1113.

Garner DL, Johnson LA, Allen CH, Palencia DD, Chambers CS, 1996b: Comparison of Seminal Quality in Holstein Bulls as Yearlings and as Mature Sires. Theriogenology, 45:923-934.

Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE, 1997a: Fluorometric Assessments of Mitochondrial Function and Viability in Cryopreserved Bovine Spermatozoa. Biol Reprod, 57:1401-1406.

Garner DL, Thomas CA, Allen CH, 1997b: Effect of Semen Dilution on Bovin Sperm Viability as Determined by Dual-DNA Staining and Flow Cytometry. J Androl, 18:324-331.

Garner DL, Thomas CA, Allen CH, Sasser RG, 1997c: Effect of Cryopreservation on Bovine Sperm Viability as Determined by Dual DNA Staining. Reprod Domest Anim, 32:279-283.

Maxwell WM and Johnson LA, 1997: Chlortetracycline Analysis of Boar Spermatozoa After Incubation, Flow Cytometric Sorting, Cooling, or Cryoperservation. Mol Reprod Dev, 46:408-418.

Maxwell WM, Welch GR, Johnson LA, 1997: Viability and Membrane Integrity of Spermatozoa after Dilution and Flow Cytometric Sorting in the Presence or Absence of Seminal Plasma. Reprod Fertil Dev, 8:1165-78.

Parks JE, Ehrenwald E, Foote RH, 1985: Counting mammalian spermatozoa in biological fluids containing particulate matter. J. Dairy Sci.,68:2329.

Penfold LM, Garner DL, Donoghue AM, Johnson LA, 1997: Comparative Viability of Bovine Sperm Frozen on a Cryomicroscope or in Straws. Theriogenology, 47:521-530.

Songsasen N, Betteridge KJ, Leibo SP, 1997: Birth of Live Mice Resulting from Oocytes Fertilized In Vitro with Cryopreserved Spermatozoa. Biol Reprod, 56:143-152.

Szollosi J, Takacs T, Balazs M, Gaspar R, Matyus L, Szabo G, Tron L, Resli I, Dajanovich S,1986: Flow-cytometric evaluation of bull semen. 1. Objective determination of sperm cell counts in diluted semen samples. Magyar Allatorvosok Lapja, 41:459-463.

Takacs T, Szollosi J, Balazs M, Gaspar R, Matyus L, Sazabo G, Tron L, Resli I, Damjanovich S: Flow cytometric determination of the sperm cell number in diluted bull semen samples by DNA staining method. Acta. Bichim. Biophys. Hung., 22:45-57.

Takizawa S, Katoh C, Fukatsu N, Horii I, 1994: Evaluation of rat sperm viability and number by flow cytometry. Teratology, 50:40B.

Takizawa S, Katoh C, Fukatsu N, Horii I, 1995: Flow Cytometric Analysis for the Evaluation of the Rat Sperm Viability and Number in the Male Reproductive Tocicity Studies. Cong. Anom, 35:177-187.

Takizawa S, Katoh C, Inomata A, Horii I, 1998: Flow cytometric analysis for sperm viability and counts in rats treated with trimethylphosphate or pyridoxine. J. Toxicol. Sci. 23:15-23. Thomas CA, Garner DL, DeJarnette JM, Marshall CE, 1997: Fluorometric Assessments of Acrosomal Integrity and Viability in Cryopreserved Bovine Spermatozoa. Reprod Biol, 56:991-998.

Thomas CA, Garner DL, DeJarnette JM, Marshall CE, 1998: Effect of Cryopreservation on Bovine Sperm Organelle Function and Viability As Determined by Flow Cytometry. Biol Reprod, 58:786-793.

Vetter CM, Miller JE, Crawford LM, Armstrong MJ, Clair JH, Conner MW, Wise DL, Skopek TR, 1998: Comparison of motility and membrane integrity to assess rat sperm viability. Reprod Toxicol, 12:105-114.

Woelders H, 1990: Overview of in vitro methods for evaluation of semen quality. Boar Semen Preservation II, Reprod. Domest. Anim, Suppl 1: 145-164.

Yamamoto T, Mori S, Yoneyama M, Imanishi M, Takeuchi M, 1996: Estimation of rat sperm with flow cytometer (FCM): simultaneous analysis of sperm number and sperm viability. Teratology, 54:38A.

Chalah T, Brillard JP, 1998: Comparison of assessment of fowl sperm viability by eosin-nigrosin and dual flourescence (SYBR-14/PI). Theriogenology, 50:487-493.

CLAIMS

1. A method for the determination of the concentration of sperm cells in a semen sample and the percentage of live sperm cells therein, comprising subjecting the semen sample or an aliquot of a diluted subsample of the semen sample to selective staining of live and dead sperm cells and combination with an internal concentration standard means and determining the concentration of the sperm cells and the percentage of live sperm cells simultaneously by means of a detection means responsive to the selective staining and the internal concentration standard means.

2. A method according to claim 1, wherein the internal concentration standard means is constituted by standardisation particles, the standardisation particles being added in a predetermined number.

3. A method according to claim 2, wherein the dilution of the sample has been performed using a diluent which sustains viability of the sperm cells during the determination.

4. A method according to any of the preceding claims, wherein the selective staining and the determination further permits determination of the percentage of dying cells (cells with a leaking membrane).

5. A method according to any of the preceding claims, wherein the selective staining is performed using one or more fluorochromes resulting in fluorescent qualities being conferred to live cells and dead cells and, where applicable, to dying cells, the fluorescent quality or qualities of live cells being distinguishable, by the determination means, from the fluorescent quality or qualities of dead cells (or, where applicable, dying cells), the standardisation particles being fluorescent particles having a fluorescent quality distinguishable from the fluorescent qualities of the live and dead or dying cells, the determination being performed by selective counting of cells/particles of each fluorescent quality.

6. A method according to claim 5, wherein the detection means comprise fluorescent activated cell sorter, such as a flow cytometer.

7. A method according to claim 5, wherein the detection means comprise fluorescent activated cell sorter, such as a laser scanning cytometer.

8. A method according to any of claims 5-7, wherein the fluorochromes are fluorochromes binding to DNA.

9. A method according to claim 8, wherein the fluorochrome staining the dead or dying cells is a fluorochrome capable of entering a cell through a leaking or defect plasma membrane, but substantially incapable of entering a cell having an intact plasma membrane, and the other fluorochrome is a fluorochrome capable of entering a cell through an intact cell membrane.

10. A method according to any of claims 5-9, wherein the excitation of the fluorochromes is performed by means of light in the wavelength range about 488 nm, the fluorochrome staining the live cells is SYBR-14, and the fluorochrome staining the dead or dying cells is PI.

11. A method according to any of claims 5-9, wherein the excitation of the fluorochromes is performed by means of light in the wavelength range about 543 nm, the fluorochrome staining the live cells is MPR71292, and the fluorochrome staining the dead or dying cells is ethidium-homodimer-2, EHD2.

12. A method according to any of claims 5-11, wherein the fluorochrome staining the live cells is used in concentrations below standard concentrations conventionally applied for such fluorochromes.

13. A method according to any of claims 5-12, wherein the flurochrome staining the live cells is used in concentrations in the range from 25 to 75 nanomolar.

14. A method according to claim 13, wherein the fluorochrome staining the live cells is used in concentrations about 50 nanomolar.

15. A method according to any of claims 5-14, wherein the staining of the cells is performed at a temperature below 35°C.

16. A method according to any of claims 5-15, wherein the staining of the c lls is performed at a temperature between 15°C and 25°C.

17. A method according to any of claims 5-16, wherein the staining of the cells is performed at room temperature.

18. A method according to any of the preceding claims, wherein the standardisation particles are fluorescent beads.

19. A method according to any of the preceding claims, wherein the size and concentration of the subsample are adapted so that the number of sperm cells corresponds to between one tenth and ten times the number of standardisation particles.

20. A method according to claim 19, wherein the size and concentration of the subsample are adapted so that the number of sperm cells corresponds to between one quarter and four times the number of standardisation particles.

21. A method according to claim 20, wherein the size and concentration of the subsample are adapted so that the number of sperm cells corresponds to between half and twice the number of standardisation particles.

22. A method according to any of the preceding claims, wherein the diluent is a diluent containing protein.

23. A method according to claim 22, wherein the protein is BSA.

24. A method according to claim 23, wherein the diluent is a diluent containing polyvinylalcohol.

25. A method according to any of the preceding claims, wherein the determination of the concentration of the sperm cells and the percentage of live sperm cells are determined as a mean value of the determination of the concentration of the sperm

cells and the percentage of live sperm cells performed at two or more subsamples of the semen sample.

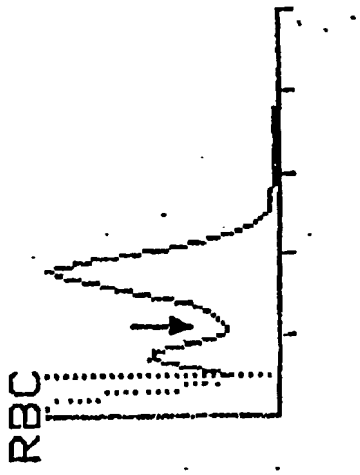

Fig. 1

Fig. 1

Fig. 2

Fig. 2

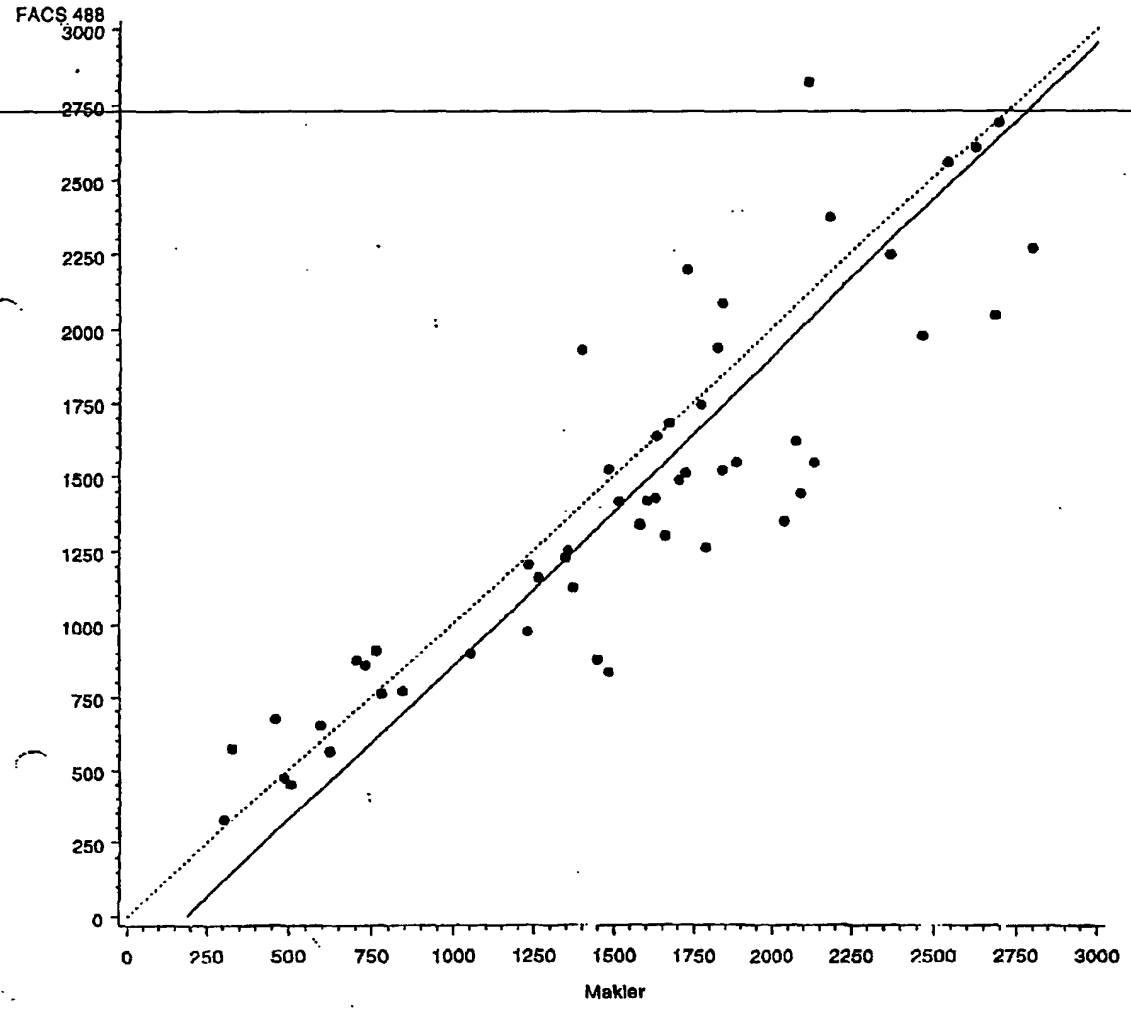

Fig. 3

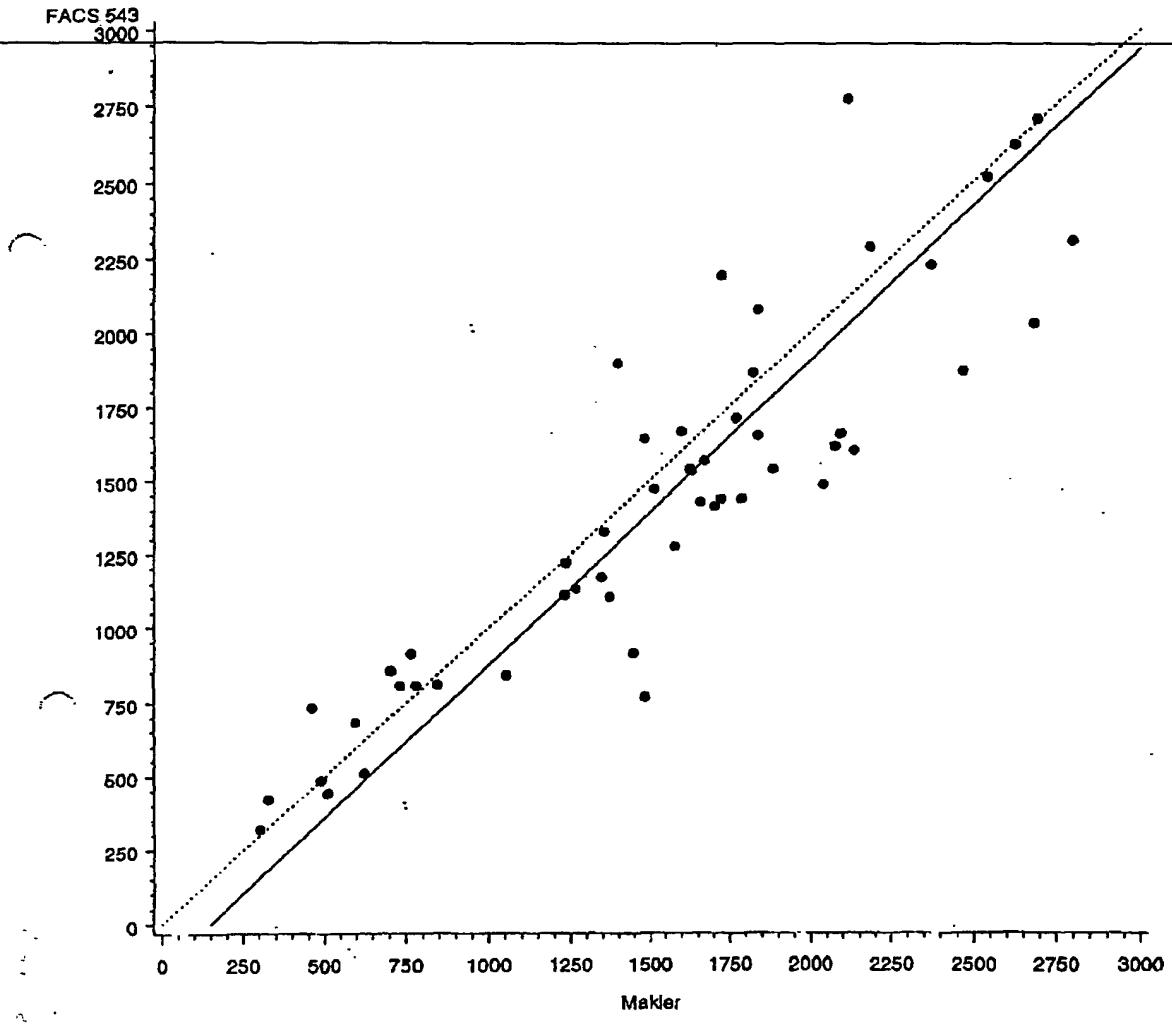

Fig. 4

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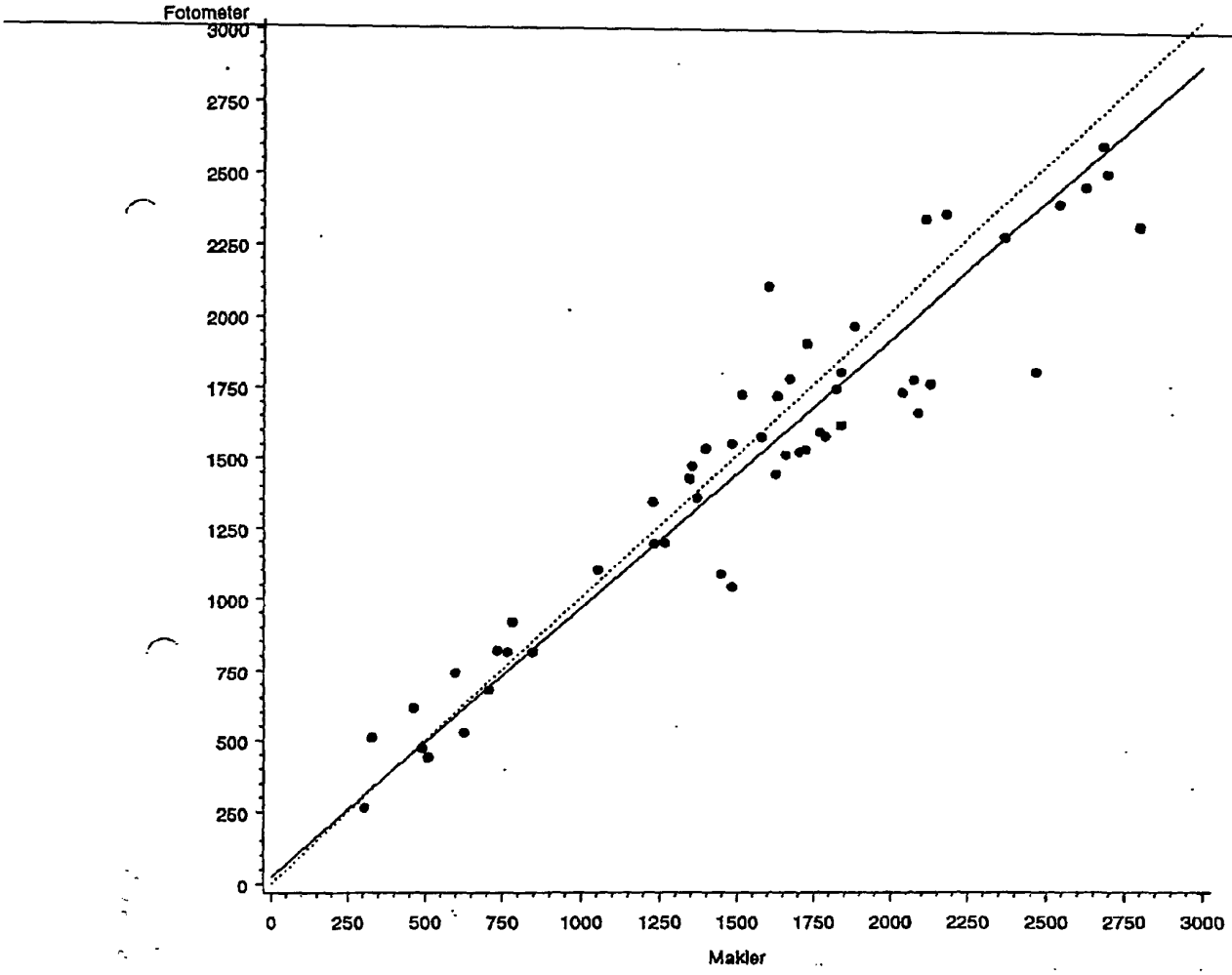

Fig. 5

Fig. 5

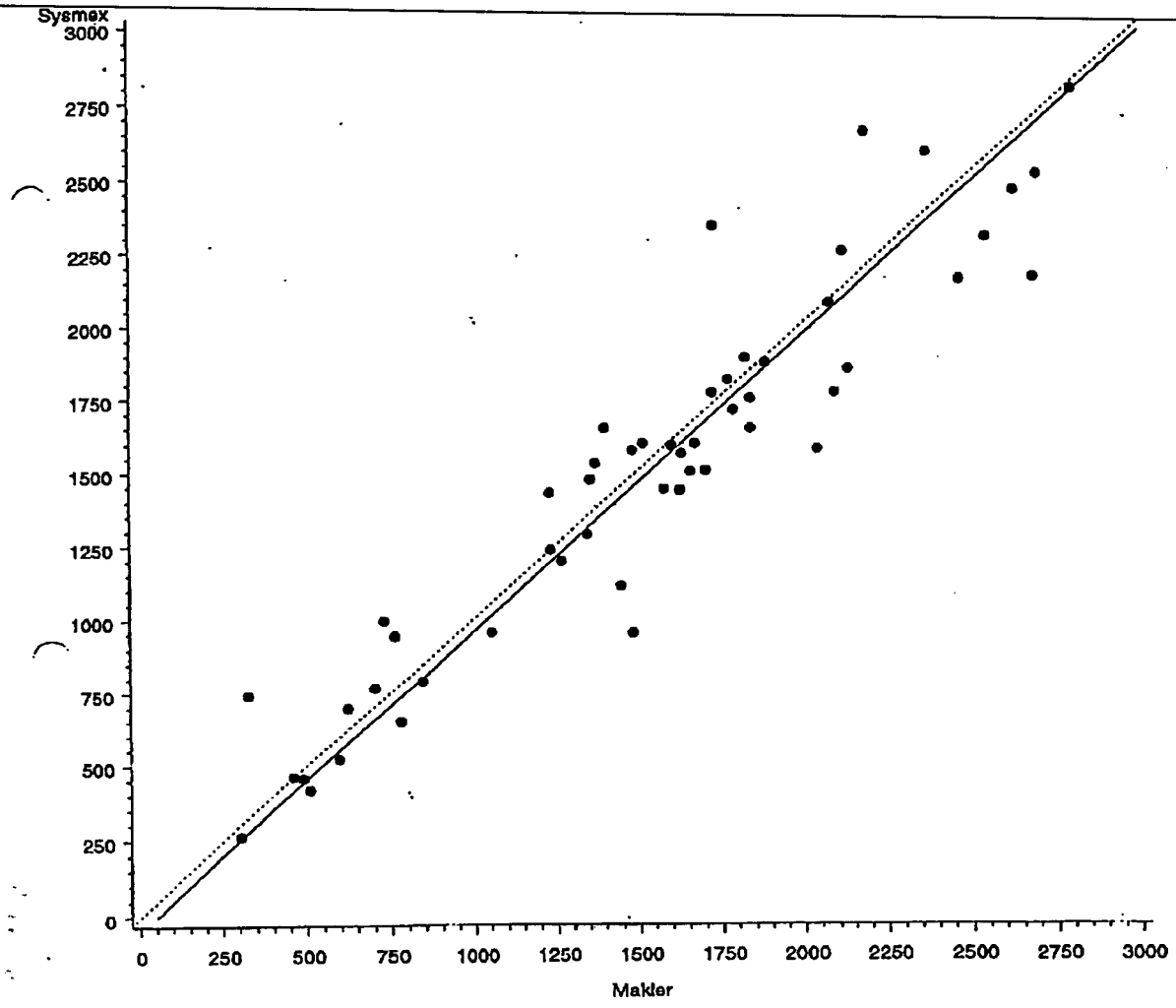

Fig. 6

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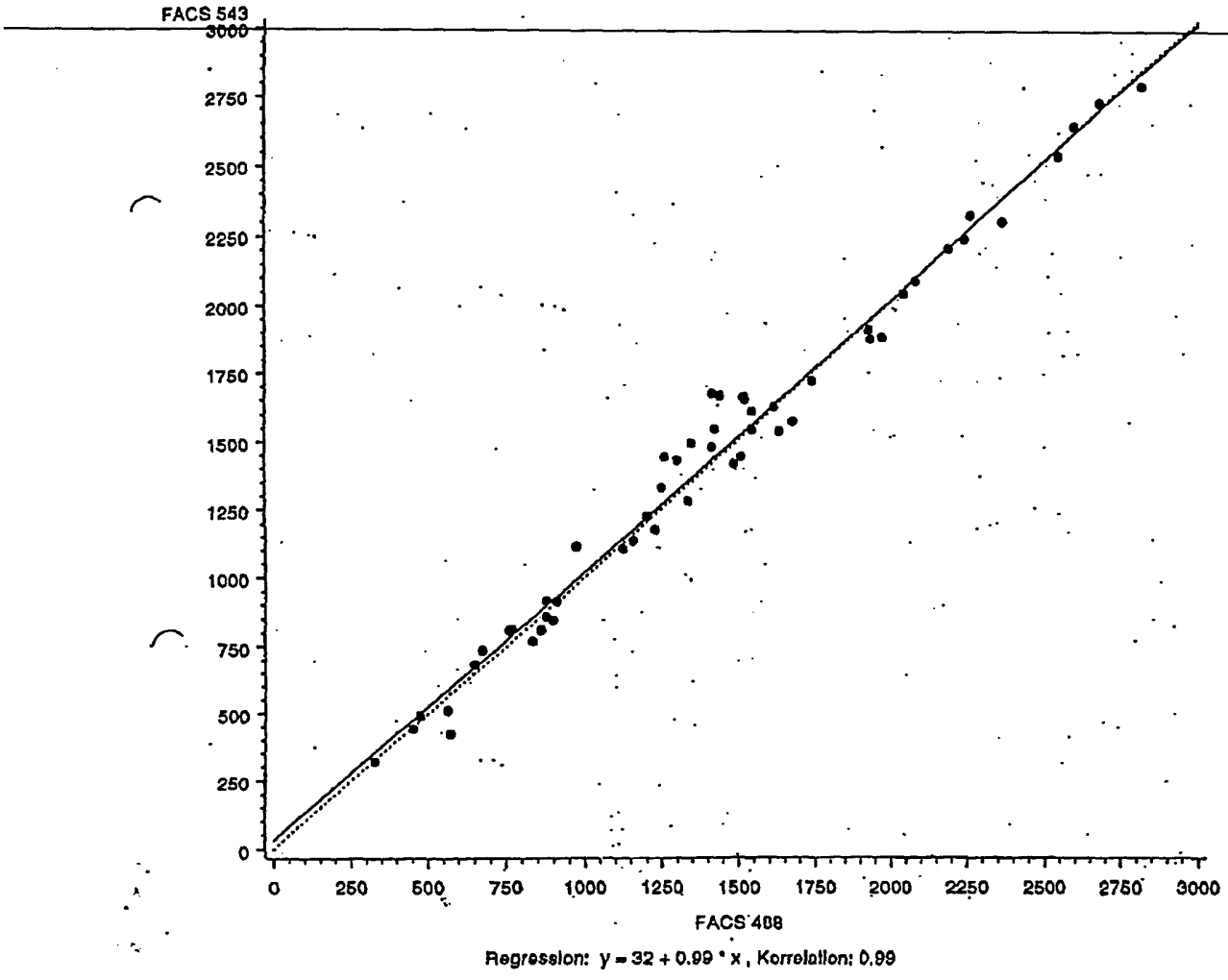

Fig. 7

Fig. 7